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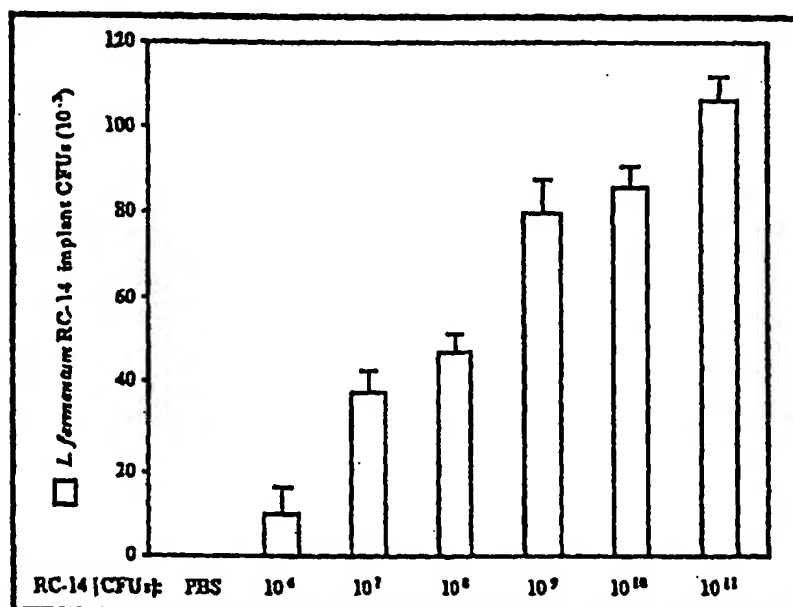
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(54) Title: **LACTOBACILLUS COMPOSITIONS AND METHODS FOR PREVENTING WOUND INFECTIONS AND BIOFILM FORMATION ON IMPLANTABLE SURGICAL DEVICES**



(57) Abstract: This invention relates to probiotic compositions and their by-products and methods of employing said compositions for inhibiting, treating or preventing wound infections and biofilm formation on implantable surgical devices.

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LACTOBACILLUS COMPOSITIONS AND METHODS FOR PREVENTING  
WOUND INFECTIONS AND BIOFILM FORMATION ON IMPLANTABLE  
SURGICAL DEVICES

5                   This invention relates to probiotic  
compositions and methods of employing said compositions  
for treating and preventing wound infections and biofilm  
formation on implantable surgical devices.

10                  Wound infections represent a catchall category  
for a group of diverse anatomic problems ranging from  
superficial and cutaneous to infections involving tissue  
and muscle invasion and foreign implants. Wound  
infections are caused by accidental (e.g. burns) and  
intentional (e.g. surgical) trauma, nosocomial  
15                  complications of surgery, hospitalization and insertion  
of implants. Wound infections are also caused by  
occupational and recreational activities. The infection-  
causing organisms (e.g. bacteria, viruses, yeasts) come  
from the patient, the hospital environment and a diverse  
20                  microbial biosphere. Infections associated with wounds  
at the skin-material interface are particularly difficult  
to prevent and treat.

                  The primary causes of wound infections are  
*Bacteroides sp.*, *Enterococcus faecalis* (vancomycin-  
25                  resistant - VRF are particularly problematic), *S. aureus*  
(including methicillin-resistant - MRSA), *S. epidermidis*  
(including methicillin-resistant) - MRSE), *Streptococcus*  
*pyogenes*, *Clostridium sp.*, *Escherichia coli*, *Pseudomonas*  
*aeruginosa*, *Klebsiella sp.*, *Proteus sp.*, and  
30                  *Peptostreptococcus sp.* (Blatan, et al. (1996) "Ten Year  
Experience with the use of ofloxacin in the treatment of  
wound infection," Antibiotiki i Khimioterapiia; DiRosa,  
et al. (1996) "Anaerobic bacteria in postsurgical

infections: isolation rate and antimicrobial susceptibility," J. Chemother., 8:91-95; Mehta, et al. (1996) "Contaminated wounds: infection rates with subcutaneous sutures," Annals Emerg. Med., 27:43-48; 5 Mousa, (1997) "Aerobic, anaerobic and fungal burn wound infections," J. Hosp. Infect., 37:317-323; Shinigawa, et al. (1997) "Bacteria isolated from surgical infections and their susceptibilities to antimicrobial agents. Special references to bacteria isolated between July 10 1995 and June 1996," Japanese J. Antibiotics, 50:143-177; Emmerson (1998) "A microbiologist's view of factors contributing to infection," New Horizons 6(2 Suppl.), S3-10). Few studies have been undertaken to examine bacterial growth and spread or to investigate the 15 infecting strains within biofilms.

The increasing emergence of multi-drug resistant organisms reduces treatment options and places the patient's life in danger. In 1992, Harold Neu wrote in Science, 257:1064-1073 about "The crisis in antibiotic 20 resistance". Since then, studies have further shown that antibiotic resistance is on the increase. This is epitomized by two extreme examples: the finding of massive increases in *E. coli* resistance to trimethoprim/sulfamethoxazole, the most commonly 25 prescribed antibiotic against simple urinary tract infection, (see Reid G., et al. (1997) "Drug resistance amongst uropathogens isolated from women in a suburban population: laboratory findings over 7 years," Can. J. Urol, 4:432-437; Gupta K., et al. (1999) "Increasing 30 prevalence of antimicrobial resistance among uropathogens causing acute uncomplicated cystitis in women", JAMA 281:736-738) and in the isolation of a new strain of

multi-drug resistant *S. aureus*, containing methicillin and vancomycin resistance genes. The latter strain represents a "superbug" capable of causing severe morbidity and death amongst patients with wounds.

5           Microbial biofilms, defined as an accumulation of microorganisms and connecting extracellular products on a surface or to each other at some distance away from a surface, (see Costerton GD, et al. (1989), "Microbial and foreign body factors in the pathogenesis of medical  
10 device infections In: Infections associated with indwelling medical devices. Bisno AL, Waldvogel FA (eds), ASM, Washington, pp. 27-59; Reid, et al. (1998), Microbial biofilms and urinary tract infections, W. Bromfitt, T. Hamilton-Miller and R.R. Bailey (eds),  
15 Chapman and Hall, London, pp. 111-118) have been found extensively in nature and in the environment in places ranging from food fermentors to oil well drilling pipes, ship hulls. Microbial biofilms are commonly associated with human disease.

20           For biofilms associated with infectious diseases, accurate prevalence and incidence figures are not available. However, microbial biofilms have been associated with many conditions including dental plaque, upper respiratory infections, peritonitis, urogenital  
25 infections, and diseases associated with surgically implanted medical devices. Dankert et al., (1986), "Biomedical polymers: bacterial adhesion, colonization, and infection," CRC Crit. Rev. Biocompat., 2:219-301; Costerton, et al. (1987), "Bacterial biofilms in nature  
30 and disease", Ann. Rev. Microbiol., 41:435-464; Bisno, et al., (1989), "Infections associated with indwelling medical devices. (eds), ASM, Washington, pp. 3-26; Blake,

et al. (1989), "Aggregation by fragilis and non-fragilis bacteroides strains, J. Med. Microbiol., 28: 9-14; Kowalewska-Grochowska, et al. (1991), "Guidewire catheter change in central venous catheter biofilm formation in a burn population", Chest, 100:1090-1095; Elliott, et al. (1992), "Infections and intravascular devices, British J. Hospital Med., 48:496-503; Bos, et al. (1996), "Co-adhesion of oral microbial pairs under flow in the presence of saliva and lactose, J. Dent. Res., 75:809-815; Reid, et al., (1998), "Microbial biofilms and urinary tract infections", In, Urinary tract infections, W. Brumfitt, T. Hamilton-Miller, and R.R. Bailey (eds), Chapman and Hall, London, pp. 111-118. Microbial biofilms have also been associated with a total artificial heart. (Jarvik, (1981), "The total artificial heart, Sci. Am., 244:74).

Surgically implanted medical devices, such as heart valves and artificial veins and joints, are especially vulnerable to microbial biofilm formation and disease. Gristina, (1987), "Biomaterial-centered infection: microbial adhesion versus tissue integration", Science, 237:1588-1595. The surfaces of such devices are not protected by host defenses and thus provide a focal point for infecting pathogens. Closed implants are frequently associated with life-threatening infections, with *Staphylococcus epidermidis* and *S. aureus* constituting the major pathogenic force, Costerton, et al. (1989), supra.

When such organisms exist as biofilms, as is the case in 80% of all infections and 850,000 catheter-associated infections in North America each year, treatment is extremely difficult. Few studies have been

undertaken to examine bacterial growth and spread or to investigate effective methods to prevent, treat or otherwise reduce the occurrence of infecting strains within biofilms.

5               Surgical infections are a great source of human morbidity and mortality. Clinical studies have shown that, under optimal circumstances, the surgical infection rate in clean wounds (i.e., Class 1 wounds) may be as low as 0.01 %. This percentage however, rapidly increases  
10               with increasing contamination of the surgical site. This is particularly true when the surgical site is complicated by the implantation of a surgical device such as an osteosynthesis plate or soft tissue alloplast augmentation. In the absence of appropriate autogenous  
15               materials to achieve the surgical goal of restoring normal anatomical relationships and physiologic functioning, surgeons are increasingly forced to use alloplastic implant material to support increasingly complicated organism trauma.

20               To combat the significant infectious complication rates associated with the use of surgical implants, several strategies have been employed. Thus far, these have been mainly limited to: improved surgical techniques; improved regimens of administering peri-  
25               operative systemic antibiotics; local antibiotic irrigation procedures; modified surface characteristics of surgical implants; and impregnating surgical implants with antibiotics.

30               Despite such strategies, little progress has been made to effectively prevent, treat or reduce the occurrence of wound infections and infections resulting from surgical implantation of medical devices.

Current citations in the literature utilize traditional methods of management, such as use of antibiotics, antiseptics and/or surgical debridement. Such methods have failed to impact infection and fatality rates.

5           We have shown previously that non-pathogenic bacteria cannot only prevent pathogenic colonization of tissues and biomaterials, but also displace these organisms. Millsap, et al., (1994), "Adhesion and displacement of *Enterococcus faecalis* by *Lactobacillus* and *Streptococcus* sp. from hydrophobic and hydrophilic substrata as studies in a parallel plate flow chamber. Appl. Environ. Microbiol. 60:1867-1874; Velraeds, et al. 10 (1996), "Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates" Appl. Environ. Microbiol. 15 62:1958-1963; Velraeds MC, et al. (1998), "Interference in initial adhesion of uropathogenic bacteria and yeasts silicone rubber by a *Lactobacillus acidophilus* biosurfactant" J. Med. Microbiol. 49:790-794. The 20 artificial implantation of such non-pathogens is referred to as probiotics. *Lactobacillus* bacteria are found in the intestine and urogenital tract where they are part of the normal, healthy flora: they represent one example of probiotics. The use of other organisms, such as avirulent 25 skin flora, such as staphylococci, can also be applied to reduce the risk of wound infections.

30           In Western and Asian society per se and clinical practice specifically, there is a slow but definite trend emerging towards the avoidance, where possible, of antibiotics and the use of naturally occurring substances for disease prevention. Studies have shown that lactobacilli not only inhibit pathogen



colonization and binding to cells and materials, but also  
displace these pathogens. The present invention applies  
this knowledge to a new area, namely the site of wounds,  
where the environment involves skin, implanted devices  
5 such as drainage tubes, hip joints, catheters, lines, and  
other prostheses.

The present invention provides methods and  
compositions for the use of *Lactobacillus* and associated  
by products including biosurfactants, avirulent  
10 staphylococci and other probiotic organisms, to prevent,  
treat, inhibit or reduce the risk of infections around  
wounds and at the site of implants. The present  
invention demonstrates that lactobacilli with  
antagonistic properties against pathogens provide  
15 protection against infection when applied topically or  
after oral intake.

In the practice of the compositions and methods  
of the present invention, the *Lactobacillus* may be  
administered as viable whole cells. The *Lactobacillus*  
20 species may be aerobically grown or microaerophilically  
grown and selected from *Lactobacillus casei*, *L.*  
*acidophilus*, *L. plantarum*, *L. fermentum*, *L. brevis*, *L.*  
*jensenii*, *L. crispatus*, *L. rhamnosus*, *L. reuteri*, *L.*  
*paracasei*, *L. gasseri*, *L. cellobiosis*, *L. delbruckii*, *L.*  
25 *helveticus*, *L. salvarius*, *L. collinoides*, *L. buchneri*, *L.*  
*rogosae* and *L. bifidum*.

The present invention provides a method for  
inhibiting the occurrence of wound infections in a mammal  
by administration of a probiotic organism. In a preferred  
30 embodiment the probiotic organism is a *Lactobacillus*. In  
a most preferred embodiment, the *Lactobacillus* species

are *L. rhamnosus* GR-1, *L. fermentum* RC-14 and *L. fermentum* B-54.

In another embodiment, the present invention provides a method for inhibiting the occurrence of wound infections in a mammal in need of such treatment by administration of *Lactobacillus* and a prebiotic compound.

In another embodiment, the present invention describes a method for inhibiting or reducing the occurrence of biofilm formation in a mammal by coating at least part of a surgically implantable device with a therapeutically effective amount of a probiotic organism or a by product thereof including biosurfactant. In a preferred embodiment the probiotic organism is a *Lactobacillus*. In a most preferred embodiment, the *Lactobacillus* species are *L. rhamnosus* GR-1, *L. fermentum* RC-14 and *L. fermentum* B-54.

In still another embodiment the present invention provides a pharmaceutical composition suitable for inhibiting the occurrence of wound infections in mammals which comprises a therapeutically effective amount of a probiotic organism and a pharmaceutically acceptable carrier. In a preferred embodiment the probiotic organism is a *Lactobacillus*. In a most preferred embodiment, the *Lactobacillus* species are *L. rhamnosus* GR-1, *L. fermentum* RC-14 and *L. fermentum* B-54.

Figure 1A is a bar graph showing the effect of *L. fermentum* RC-14 on surgical implant infection in Sprague Dawley rats. Surgical implants removed from RC-14 treated rats (see Table 2) were rinsed in PBS and then briefly sonicated (30 sec) to recover implant associated bacteria. PBS diluted bacterial suspensions ( $10^{-3}$ ) were then plated onto MRS agar plates and incubated overnight

at 37°C in an anaerobic (5% CO<sub>2</sub>) bacterial chamber. Colonies were then scored for each of the 7 groups of inoculated rats (9 rats/group).

Figure 1B is a bar graph showing the effect of *L. rhamnosus* GR-1 on surgical implant infection in Sprague Dawley rats. Surgical implants removed from GR-1 treated rats (see Table 2) were rinsed in PBS and then briefly sonicated (30 sec) to recover implant associated bacteria. PBS diluted bacterial suspensions (10<sup>-3</sup>) were then plated onto MRS agar plates and incubated overnight at 37°C in an anaerobic (5% CO<sub>2</sub>) bacterial chamber. Colonies were then scored for each of the 7 groups of inoculated rats (9 rats/group).

Figure 2 is a surface enhanced laser desorption/ionization (SELDI) mass profile of lactobacillus expression of collagen binding proteins in *Lactobacillus acidophilus* RC-14; *L.rhamnosus* GR-1 and *L. rhamnosus* 36W.

Figure 3 is a SELDI mass profile of lactobacillus expression of collagen binding proteins in *Lactobacillus acidophilus* RC-14 using protein chip PS-1/CN-III.

Figure 4 is a bar graph illustrating dose dependant *S. aureus* rates and associated *S. aureus* surgical implant colony forming units (CFUs). Silicone implants (1cm<sup>2</sup>) placed within a surgically subcutaneous pocket located on the dorsum of male rats (Sprague Dawley, 300gm) were inoculated with the indicated number of colony forming units (CFUs) of *S. aureus*. Animals were sacrificed 3 days later. Acute infection rates were scored and the number of *S. aureus* [CFUs] per implant

determined. The 50% infectious dose (ID<sub>50</sub>) for *S. aureus* in this *in vivo* model was determined to be  $\sim 7 \times 10^6$  CFUs.

Figure 5A is a bar graph illustrating the effect of co-inoculation of *S. aureus* (ID<sub>50</sub>%) and *L. fermentum* RC-14 on the incidence of surgical implant infection in Sprague-Dawley rats. Surgical implants were recovered from rats co-inoculated with *S. aureus* [ID<sub>50</sub>] and the indicated number of RC-14 CFUs 3 days after surgery (see Table 2). Implants were rinsed in PBS and then briefly sonicated (30 sec) to recover implant associated bacteria. The indicated dilutions of the implant associated bacterial suspensions were then plated on either MRS (RC-14) or BH agar (*S. aureus*) plates and incubated overnight at 37°C. MRS plates were incubated in a specific anaerobic (5% CO<sub>2</sub>) chamber. Colonies were then scored for each of the indicated co-inoculated groups of rats (9 rats/group).

Figure 5B depicts the results of PCR-based identification of implant associated RC-14 CFUs from *S. aureus* and RC-14 co-inoculated rats. Surgical implant associated *L. fermentum* RC-14 CFUs were positively identified using RC-14 specific primers that were designed to amplify a specific portion ( $\sim 100$ bp) of the 16S-23S rDNA intergenic spacer region. To control for the possibility that the RC-14 scored CFUs are *S. aureus* positive PCR was performed on genomic DNA extracted from several single MRS colonies using both *S. aureus* and RC-14 specific primers. PCR identification of the *S. aureus* species was performed using *Staphylococcus* specific 16S-23S rDNA intergenic spacer primers (Mendoza et al. (1998) International Journal of Systematic Bacteriology 48:1049-

1055, incorporated herein by reference). ND\* = no DNA  
(negative control reaction), M = 1 Kb DNA ladder marker.

Figure 5C depicts the results of PCR-based  
identification of implant associated *S. aureus* CFUs from  
*S. aureus* and RC-14 co-inoculated rats. Surgical implant  
associated *S. aureus* CFUs were positively identified  
using *Staphylococcus* specific 16S-23S rDNA intergenic  
spacer primers (see Mendoza et al. International Journal  
of Systematic Bacteriology 48:1049-1055, 1998, supra). To  
control for the possibility that the *S. aureus* scored  
CFUs were also RC-14 positive, PCR was performed on  
genomic DNA extracted from single BH-agar colonies using  
both *S. aureus* and RC-14 specific primers. M = 1 Kb DNA  
ladder marker.

Figure 6 is a bar graph showing the effect of  
Biosurfactants (BSF) isolated from probiotic strain *L.*  
*fermentum* RC-14 on *S. aureus* [ID50] induced surgical  
implant infection in rats. Silicone implants (1cm<sup>2</sup>)  
were pretreated (12 hrs, 4°C) with *L. fermentum* RC-14 BSF  
(1 mg/ml) prior to surgical placement in the animals as  
described above. The surgical site containing the  
silicone implant was then co-inoculated with *S. aureus*  
[ID50] and 100 mg of *L. fermentum* RC-14 BSF,  
respectively. Animals were sacrificed 3 days after  
surgery. Acute surgical infection was scored and the  
number of surgical implant associated *S. aureus* CFUs  
determined, as described above. \*\*P < 0.004

Figure 7 is a bar graph showing the effect of  
p29 collagen binding protein (p29CnB) on *S. aureus*  
[ID100] induced surgical implant infection in rats.  
Silicone implants (1cm<sup>2</sup>) were pretreated (12 hrs, 4°C)  
with recombinant p29CnB (His-tag) or BSA (1 mg/ml) prior

to surgical placement in the animals as described above. The surgical site containing the silicone implants were then co-inoculated with  $10^8$  CFUs of *S. aureus* [ID100] and either 100 mg of p29CnB or BSA (negative control),  
5 respectively. Animals were sacrificed 3 days after surgery. Acute surgical infection was scored and wound tissue and implants collected for further analysis.\*\*  $P < 0.05$  (Infection rate and Implant *S. aureus* CFUs).

The present invention provides methods for the  
10 inhibition of wound infections of mammals including humans which comprises administering a therapeutically effective amount of a probiotic organism or a by product thereof to a wound infection site and/or a biocompatible medical device. By "inhibition" is meant treating or  
15 reducing the occurrence of wound infections with the probiotic organisms of the present invention. By "by product" is meant biosurfactants, anti-adhesion molecules and immune modulators which exhibit the infection-inhibiting activity of probiotics.

The artificial implantation of non-pathogenic  
20 probiotics provides novel intervention strategies that reduce the risk of infections around wounds and other surgical sites. The adherence of these strains and their expression of antagonistic activity against pathogens,  
25 i.e., against adhesion, growth or ability to dominate the flora, are critical factors in competition. Such microbial competition and interference takes place at certain wound locations such as on the skin, in the oral and gastrointestinal (GI) tract and the genito-urinary  
30 tract.

By "probiotic" is meant an organism which has one or more of the following characteristics, an ability

to facilitate or enhance wound healing comprising an ability to: adhere to epidermal or epithelial cells by electrostatic, hydrophobic or specific adhesins including a collagen binding protein; pass through the stomach and reach the small and large intestine; grow and persist in the gastrointestinal, urogenital tracts and at a wound surface interfaces; inhibit the adhesion of wound-associated pathogens including organisms which cause infection; coaggregate; produce acid and other substances such as hydrogen peroxide and/or bacteriocins and bacteriocin-like compounds which inhibit pathogen growth; produce biosurfactant or related by-products of growth which interfere with adhesion of pathogens to cells and materials; resist antimicrobial agents; and/or enhance the host's immune function to further inhibit pathogen growth. A preferred probiotic bacteria is one or more species of lactobacillus or by-products thereof such as proteins or peptides or amino acids as identified using SELDI methodology.

Separation and detection of biosurfactants produced by lactobacilli may be preferably accomplished by the SELDI technique (Surface Enhanced Laser desorption/ionization). By "SELDI system" is meant a method which uses protein chips which contain chemically or biologically treated surfaces that specifically interact with or bind the proteins of interest. The protein chips are inserted into a reader which provides an accurate mass profile of the proteins bound to each chip in just a few minutes. A most preferred probiotic lactobacillus species is *L. fermentum* RC-14. Another preferred lactobacillus species is *L. rhamnosus* GR-1.

Still another preferred lactobacillus species is  
L.fermentum B-54.

The preferred strains of lactobacilli within  
the scope of this invention are anaerobic and  
5 microaerophilic isolates.

By "biosurfactant" is meant a biological  
substance, for example a protein or peptide, produced by  
a lactobacillus having a molecular weight of about 5 kd  
to about 100 kd which inhibits the binding of pathogenic  
10 bacteria to surfaces. Components of the biosurfactant  
can stimulate immune defenses of a host to promote the  
reduction and prevention of wound infections. The  
preferred lactobacillus by-product is a biosurfactant  
having a molecular weight of about 8 kd to about 90 kd.

15 By "anti-adhesin" is meant a biological  
substance which inhibits, reduces or prevents adhesion of  
pathogenic bacteria to surfaces.

By "prebiotic" is meant a nonmetabolized,  
nonabsorbed substrate that is useful for the host which  
20 selectively enhances the growth and/or the metabolic  
activity of a bacterium or a group of bacteria. A  
prebiotic also includes a nutrient utilized by  
lactobacilli to stimulate and/or enhance growth of  
lactobacilli relative to pathogenic bacteria.

25 By "infected wound" is meant an area of open  
and inflamed epidermal tissue with detectable levels of  
pathogens e.g., staphylococcus or candida, present. In  
accordance with the present invention visual inspection  
of a wound can accurately determine indicia of infection,  
30 including but not limited to, inflammation, discharge,  
the patient's subjective pain assessments, bleeding, and  
presence of dead cells, for example.



In accordance with the present invention a "biofilm" means an accumulation of microorganisms and connecting extracellular products on a surface or to each other at some distance away from a surface.

Also defined within the present invention are compositions suitable for inhibiting wound infections of mammals including humans which comprise one or more lactobacillus viable whole cells, non-viable whole cells or cell wall fragments and a pharmaceutically acceptable carrier.

In a preferred aspect, the lactobacillus is aerobically, microaerophilically or anaerobically grown and may be selected from the group consisting of *Lactobacillus casei*, *L. acidophilus*, *L. plantarum*, *L. fermentum*, *L. brevis*, *L. jensenii*, *L. crispatus*, *L. rhamnosus*, *L. reuteri*, *L. paracasei*, *L. gasseri*, *L. cellobiosus*, *L. delbruckii*, *L. helveticus*, *L. salvarius*, *L. collinoides*, *L. buchneri*, *L. rogosae* and *L. bifidum*.

The lactobacillus may be microaerophilically or anaerobically grown and selected from the group consisting of *Lactobacillus casei* var *rhamnosus* (GR-1 (ATCC 55826), *L. casei* var *rhamnosus* GR-2 (ATCC 55915), *L. casei* var *rhamnosus* GR-3 (ATCC 55917), *L. casei* var *rhamnosus* GR-4 (ATCC 55916), *L. casei* var *rhamnosus* RC-9, *L. casei* var *rhamnosus* RC-17 (ATCC 55825), *L. casei* var *alactosus* RC-21, *L. casei* NRC 430, *L. casei* ATCC 7469, *L. casei* var *rhamnosus* 81, *L. casei* var *rhamnosus* 76, *L. casei* var *rhamnosus* 36W, *L. casei* var *rhamnosus* 36g, *L. casei* RC-65, *L. casei* RC-15, *L. casei* 558, *L. casei*, RC-21, *L. casei* 55, *L. casei* 8, *L. casei* 43, *L. plantarum* RC-12 (ATCC 55895), *L. acidophilus* RC-25, *L. plantarum* RC-19, *L. jensenii* RC-11 (ATCC 55901), *L. acidophilus*

ATCC 4357, *L. acidophilus* 2099B, *L. acidophilus* 2155C, *L. acidophilus* T-13, *L. acidophilus* 1807B, *L. acidophilus* RC-16, *L. acidophilus* RC-26, *L. acidophilus* RC-10, *L. acidophilus* RC-24, *L. acidophilus* RC-13, *L. acidophilus* RC-14, *L. acidophilus* RC-12, *L. acidophilus* RC-22, *L. acidophilus* 2099B, *L. acidophilus* 2155C, *L. acidophilus* T-13, *L. plantarum* ATCC 8014, *L. plantarum* UH 2153, *L. plantarum* 260, *L. plantarum* RC-20, *L. plantarum* 75, *L. plantarum* RC-6, *L. fermentum* A-60, *L. fermentum* B-54 (ATCC 55920), *L. cellobiosus* RC-2, *L. crispatus* 1350B and *L. crispatus* 2142B.

In another aspect of this invention, a pharmaceutical composition is provided for inhibiting wound infections in humans and lower animals which comprises a therapeutically effective amount of one or more of the aforementioned lactobacilli, together with a pharmaceutically acceptable carrier.

Bacteria on surfaces, such as mucosal tissues and skin, exist in biofilms and compete with other organisms for space and nutrients.

The lactobacillus compositions of the present invention at various doses (e.g.,  $10^3$ - $10^{11}$ ) significantly reduce surgical implant infection caused by *S. aureus*, staphylococci and candida by competitively excluding such pathogens. Non-pathogenic organisms, such as viable or non-viable lactobacilli, or their by-products such as biosurfactants, anti-adhesins and immune modulators, are applied to the wound surface interface or the biocompatible device-host interface, to reduce the risk of infecting pathogens colonizing and infecting the host. The lactobacilli form a barrier to biofilm formation and infection by organisms which infect the host, for

example, opportunistic pathogens such as *S. aureus*.

Biosurfactants produced by lactobacilli significantly inhibit the binding of pathogens to wound sites. These biosurfactants contain carbohydrate and proteinaceous compounds. Biochemical analysis using PAGE, affinity chromatography, and amino acid sequencing of biosurfactant produced by *L. fermentum* RC-14 evidences a 26kD protein which binds to collagen. This protein, and others which also bind to collagen, play a role in the colonization by lactobacilli at the wound surface interface. This 26kD protein is also understood, in accordance with the present invention to play an important role in the protection of the heart against urogenital pathogens. Moreover, this 26kD protein (known in accordance with the present invention as p29 or p26 collagen binding protein (p29CnB or p26CnB)) significantly decreases the implant infection rate caused by *S. aureus*.

When more than 100,000 bacteria are present per cubic cm (per gram tissue), the killing capacity of the host's white blood cells is diminished. To effectively reduce and inhibit the occurrence of pathogenic bacteria a dosage of  $10^3$  to about  $10^{11}$  viable or non-viable lactobacilli per ml, and optionally about 0.1 to about  $10\mu\text{g/ml}$  of biosurfactant or other anti-adhesion molecule is applied directly to the wound infection site on the skin or device interface. This administration is embodied in a suitable carrier, such as a cream, paste, solution, hydrogel, liposome, for example. The treatment using the organisms is preferably administered in a single dose. For curative treatment administration of the compositions contemplated by the present invention is

from about 1 to about 3 times daily to about 1 to about 3 times weekly depending upon the severity of the infection.

5 The effect of the probiotic composition is further enhanced by stimulating lactobacilli growth over that of pathogens at a surface, using prebiotics, such as a natural sugar, inulin, extracted from chicory roots and found not to be metabolized by humans but to act as a substrate for growth of lactobacilli, for example. (See 10 Roberfroid MB (1998) British J. Nutrition, 80 suppl. 2:S197-S202, incorporated herein by reference).

15 Although this invention is not intended to be limited to any particular mode of application, topical, intravenous or oral administration of the compositions are preferred. The compositions may be administered in the form of a cream, liquid, paste, or gel as desired. One preferred form is a cream formulation comprising one or more lactobacillus viable whole cells, non-viable whole cells or in a base that is non-toxic nor irritating 20 to the skin such as a hydrogel or liposome base. For example, a contemplated cream formulation includes cocoa butter. Another preferred form of application involves the preparation of a freeze-dried capsule, taken orally comprising the composition of the present invention. It 25 has been found that a capsule comprising about  $10^3$  to about  $10^{11}$  probiotic organisms is suitable. In accordance with the present invention a capsule may contain one single or two or more different species of probiotic organism(s).

30 By "therapeutically effective amount" as used herein is meant an amount of a probiotic organism, e.g., *Lactobacillus*, high enough to significantly positively

modify the condition to be treated but low enough to avoid serious side effects (at a reasonable benefit/risk ratio), within the scope of sound medical judgment. A therapeutically effective amount of lactobacillus will vary with the particular wound infection being treated, the age and physical condition of the patient being treated, the severity of the infection, the duration of treatment, the nature of concurrent therapy and the specific lactobacillus employed. The effective amount of lactobacillus will thus be the minimum amount which will provide the desired attachment to epithelial and epidermal cells. The presence of about  $10^3$  to about  $10^{11}$  bacteria, as viable or non-viable whole cells, in 0.05 ml solution of phosphate buffered saline solution, or in 0.05 ml of suspension of broth, or the dry weight equivalent of cell wall fragments, is effective when administered in quantities of from about 0.05 ml to about 20 ml.

By "pharmaceutically-acceptable carrier" as used herein is meant one or more compatible solid or liquid filler diluents, or encapsulating substances. By "compatible" as used herein is meant that the components of the composition are capable of being comingled without interacting in a manner which would substantially decrease the pharmaceutical efficacy of the total composition under ordinary use situations.

A decided practical advantage is that the probiotic organism may be administered in a convenient manner such as by the topical, oral, intravenous (where non-viable), or suppository (vaginal or rectal) routes. Depending on the route of administration, the active ingredients which comprise probiotic organisms may be

required to be coated in a material to protect said organisms from the action of enzymes, acids and other natural conditions which may inactivate said organisms. In order to administer probiotic organisms by other than  
5 parenteral administration, they should be coated by, or administered with, a material to prevent inactivation. For example, probiotic organisms may be co-administered with enzyme inhibitors or in liposomes. Enzyme inhibitors include pancreatic trypsin inhibitor,  
10 diisopropylfluorophosphate (DFP) and trasylol. Liposomes include water-in-oil-in-water P40 emulsions as well as conventional and specifically designed liposomes which transport lactobacilli or their by-products to the urogenital surface.

15 The probiotic organisms may also be administered parenterally or intraperitoneally. Dispersions can also be prepared, for example, in glycerol, liquid polyethylene glycols, and mixtures thereof, and in oils.

20 The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be  
25 sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like),  
30 suitable mixtures thereof and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the

required particle size in the case of dispersion. In many cases it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the probiotic organisms in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized probiotic organisms into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof. Additional preferred methods of preparation include but are not limited to lyophilization and heat-drying.

When the probiotic organisms are suitably protected as described above, the active compound may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets designed to pass through the stomach (i.e., enteric coated), or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the probiotic organisms may be

incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains about  $10^3$  to about  $10^{11}$  viable or non-viable e.g., lactobacilli per ml.

The tablets, troches, pills, capsules or lactobacilli in suspension as described above, may also contain the following: a binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid, and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavoring agent such as peppermint, oil or wintergreen or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills or capsules or lactobacilli in suspension may be coated with shellac, sugar or both.

A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the probiotic organism may



be incorporated into sustained-release preparations and formulations.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of the probiotic organisms calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly depending on (a) the unique characteristics of the probiotic organism and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such probiotic for the establishment and maintenance of a healthy urogenital flora.

The probiotic organism is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically or food acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active compound in an amount approximating  $10^3$  to about  $10^{11}$  viable or non-viable lactobacilli, per ml. In the case of compositions containing supplementary ingredients such as prebiotics, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

The pharmaceutically acceptable carrier may be in the form of milk or portions thereof including yogurt. Skim milk, skim milk powder, non-milk or non-lactose

containing products may also be employed. The skim milk powder is conventionally suspended in phosphate buffered saline (PBS), autoclaved or filtered to eradicate proteinaceous and living contaminants, then freeze dried heat dried, vacuum dried, or lyophilized.

Some other examples of substances which can serve as pharmaceutical carriers are sugars, such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethylcellulose, ethylcellulose and cellulose acetates; powdered tragacanth; malt; gelatin; talc; stearic acids; magnesium stearate; calcium sulfate; calcium carbonate; vegetable oils, such as peanut oils, cotton seed oil, sesame oil, olive oil, corn oil and oil of theobroma; polyols such as propylene glycol, glycerine, sorbitol, manitol, and polyethylene glycol; agar; alginic acids; pyrogen-free water; isotonic saline; cranberry extracts and phosphate buffer solution; skim milk powder; as well as other non-toxic compatible substances used in pharmaceutical formulations such as Vitamin C, estrogen and echinacea, for example. Wetting agents and lubricants such as sodium lauryl sulfate, as well as coloring agents, flavoring agents, lubricants, excipients, tableting agents, stabilizers, anti-oxidants and preservatives, can also be present.

Accordingly, in a preferred form of treatment of wound infections, the patient is administered a therapeutically effective amount of a lactobacillus composition in accordance with the present invention, for example, topically.

A most preferred composition comprises one or more lactobacillus viable whole cells and a

pharmaceutically acceptable carrier. This composition may be administered topically, orally or intravenously in the form of a cream, capsule, gel, liquid or paste.

Preferably, the lactobacillus is selected from the group comprising *L. casei ss rhamnosus*, *L. casei ss alactosus*, *L. fermentum* and *L. brevis*. Most preferably, the lactobacillus is either *L. rhamnosus* GR-1, *L. fermentum* B-54 or *L. fermentum* RC-14.

In a further aspect of this invention, a method of treating or preventing biofilm formation is provided which involves coating a surgically implantable device with a therapeutically effective amount of one or more of lactobacillus viable whole cells, non-viable whole cells, or a cell wall fragment.

The administration of lactobacilli probiotics to a wound site and subsequent production of anti-pathogenic products by the lactobacilli (e.g., biosurfactants, acids, hydrogen peroxide, bacteriocins) stimulates the immune response against infection and reduces the risk of medical device associated infections. While not wishing to be bound by a particular mechanism, host responses are stimulated which inhibit pathogens and/or create a microenvironment less conducive to pathogen spread. Accordingly, in a preferred embodiment of stimulating host responses, a medical device is contacted or coated with lactobacillus at a concentration of about  $10^3$  to about  $10^{11}$  organisms/ml prior to introduction into a patient in need of such device.

The surgically implantable device may be composed of polymers such as fluorinated ethylene propylene, sulfonated polystyrene, polystyrene, polyvinyl chloride (PVC), polyurethane or polyethylene terephthalate

silicon rubber or other biomaterials and in addition,  
glass or hydrophilic substrata. The devices contemplated  
by the present invention include, but are not limited to,  
artificial heart valves, artificial veins, artificial  
5 arteries, shunts, drainage tubes, joints or catheters,  
intrauterine devices, catheters, stents, intravenous  
lines, diaphragms, implants, screws, sutures, pads and  
tampons, for example.

Although the present invention is not bound by  
10 any one theory or mode of operation, it is believed that,  
at least to some degree, a combination of coaggregation of  
lactobacillus and the production by lactobacillus of one  
or more inhibitory substances is responsible for excluding  
pathogens and/or reducing their numbers at the wound site.  
15 For example, a collagen binding protein is contemplated to  
transduce a signal which interferes with pathogen  
virulence. The signal transduced by the collagen binding  
protein causes the pathogen to rapidly become avirulent or  
aseptic.

20 Thus, the present invention contemplates methods  
for inhibiting pathogenic colonization in a host at a  
wound-surface interface, for example, by the  
administration of a probiotic cultured to induce the  
production of a collagen binding protein.

25 From the standpoint of physical exclusion, the  
attachment of lactobacillus acts as a block to pathogens  
by preventing access to receptor sites. Although complete  
exclusion of pathogens theoretically can occur, the most  
common finding of the results of the present invention is  
30 that there is a reduction in pathogen numbers compared to  
lactobacilli. In other words, although some lactobacilli

may not completely exclude pathogens, they are still capable of interfering with pathogen colonization in vivo.

The following examples are intended to further illustrate the invention.

5

**EXAMPLE 1****Animal Housing**

Eight week-old (300 gram) male Sprague-Dawley rats (Charles River Inc, Montreal QC) were housed in shoebox plastic cages (2 animals per cage) in the animal facilities of the Lawson Research Institute, maintained on a 12-hour light cycle, and given free access to standard rodent chow and water. Following surgery, the animals were examined daily for clinical signs of infection.

**Bacterial Cultures**

*Staphylococcus aureus* (Oxford strain) was cultured in Brain Heart (BH) growth media overnight at 37°C and plated on BH agar culture plates to determine the colony-forming unit (CFU) activity of the bacteria. Following CFU measurements *Staphylococcus aureus* (SA) suspensions were diluted in phosphate buffered saline (PBS) to attain the correct number of CFUs for subsequent animal inoculations. In a similar procedure, *Lactobacillus fermentum* RC-14 and *Lactobacillus rhamnosus* GR-1 were prepared using MRS Broth and MRS agar culture plates. Appropriate CFU measurements and inoculation dilutions were prepared as described above for *S. aureus* (SA).

**Biosurfactant Production and isolation**

Crude biosurfactant (BSF) was collected from *Lactobacilli* strains GR-1 and RC-14 as previously described (Reid et al. Methods in Enzymology 310:426-433, 1999, incorporated herein by reference). Briefly, bacterial cells were harvested by centrifugation (10,000 x g, 10 min, 10°C), washed twice in demineralized water and suspended in PBS. The *Lactobacilli* were placed at room

temperature for biosurfactant release with gentle stirring (2 hr). The bacteria and surfactant were separated by centrifugation (10,000 x g, 10 min, 10°C), and the supernatant was removed, filtered (0.22µm) and dialyzed against double demineralized water at 4°C using spectrapor dialysis tubing (6000-8000 Da M.W. cutoff). The dialyzed BSF was either used directly or freeze-dried (-10°C, -5µm Hg, 1-2 days) followed by overnight drying (Savant speedvac, RCT4104). The biosurfactant powder was stored at -20°C. Protein concentrations were determined using a protein assay kit (Pierce, ON). For surgical implant experiments the BSF was suspended in sterile PBS (2mg/ml) and incubated with the surgical implants for 12 hours at 4°C prior to placing them in the animals.

#### Animal Surgery

Sprague-Dawley rats were anaesthetized via peritoneal injection of a mixture of hydrochloride-ketamine (100 mg/ml) and xylazine (10mg/ml) at the rate of 0.1ml per 100g of body weight. Each anaesthetized rat was clipped of dorsal hair at the surgical site and liberally swabbed with a povi-iodine antiseptic solution prior to surgery. A single 2cm incision was made along the dorsal skin. A single (1cm x 1cm x 0.5 mm) sterile silicone implant (Dagnone Inc, Quebec) was then inserted into the subcutaneous pocket adjacent to the skin incision and inoculated with the indicated number of bacterial CFUs and/or BSF. The incision was closed with 3.0 coated polygalactin 910 (vicryl) interrupted sutures and a post-operative analgesic (buprenorphine hydrochloride, 0.01 mg/kg subcutaneously) was administered to each animal.

**Harvesting of implants and tissue**

At the specified post-operative times animals were sacrificed by CO<sub>2</sub> asphyxiation. Acute surgical infection was scored (large fluid-filled capsule containing the surgical implant) and the surgical implants, wound tissue and fluid harvested for further analysis. Implants from uninfected rats were free of any signs of inflammation. The implant-associated bacteria [CFUs] numbers were quantified using standard microbiological techniques. Briefly, implants were rinsed in PBS and then subjected to a 30-second sonication treatment. Diluted bacterial suspensions were then plated on either MRS- or BH-agar plates.

**Gram Staining of Implants**

Harvested implants were analyzed using a Gram staining kit (CMS protocol, Fisher) in order to assess both the extent of the biofilm on the implant and the efficiency of the sonication treatment. The staining procedure included: Crystal violet (1 min), iodine solution (1 min), decolorizing agent (30 sec), sephranin (1 min). Implants were then imaged using a light microscope (Axiophot, Zeiss).

**Polymerase Chain reaction (PCR)**

The PCR technique was used to unambiguously identify the types of bacterial CFUs obtained from each surgical implant. Briefly, bacteria colonies were picked, washed in Tris-EDTA buffer (pH 7.4), and lysed in 10% SDS for 30 minutes (Bollet et al. Nucleic Acid Research 19(8):1955 ). The lysate was isolated by brief centrifugation (4000 rpm, 5min) and heated in a 300 Watt-



microwave (Hi power) for 5 minutes. The pellets were subjected to one round of phenol/chloroform/isoamyl alcohol (24:23:1) extraction (pH 8.0) and the DNA precipitated with two volumes of absolute ethanol. The DNA was then added to a PCR reaction mixture containing the following additional reagents: 0.5mM specific oligonucleotide primers [*Staphylococcus aureus* (Jensen et al. Appl. Environ. Microbiol. 59:945-952, 1993; Mendoza et al. International Journal of Systematic Bacteriology 48:1049-1055, 1998) forward primer = 5'GAAGTCGTAACAAGG-3' (SEQ ID NO:1) and reverse primer = 5'-CAAGGCATCCACCGT-3' (SEQ ID NO:2); *L. fermentum* RC-14 forward primer = 5'-AAACTTTCTTATTCTATTCTGGT -3' (SEQ ID NO:3) and reverse primer = 5'-AACTGATTCGTCCCGTAAA-3' (SEQ ID NO:4); *L. rhamnosus* GR-1 (Tilsala-Timisjarvi and Alatossava Appl. Environ. Microbiol. 64(12):4816-4819, 1998) forward primer = 5'-ACGAGGCAC-3' (SEQ ID NO:5), reverse primer = 5'-ACGCGCCCT-3' (SEQ ID NO:6)], 0.3 mM dNTP, 2mM MgCl<sub>2</sub>, and 1 unit of Platinum Taq Polymerase (Gibco-BRL) to a total volume reaction of 50ml. PCR thermal cycling (Model 212 Lab-line) profiles used included: denaturation (2 min / 94°C), 25 amplification cycles for *S. aureus* [denaturation (1 min at 94°C), annealing (7 min at 52°C) and elongation (2 min at 68°C)] or 40 cycles for RC-14 and GR-1 [denaturation (1 min / 94°C), annealing (2 min / 52°C or 35°C, respectively) and extension (2 min at 68°C)]. The PCR products were then separated by electrophoresis on a 2% agarose gel and photographed.

**Table 1**The effects of Probiotic strains of *Lactobacilli* on  
Surgical Implants in Rats

Lactobacillus strain	Dose [CFUs] <sup>a</sup>	Infection (%) <sup>b</sup>
GR-1	10 <sup>6</sup>	0
	10 <sup>7</sup>	0
	10 <sup>8</sup>	0
	10 <sup>9</sup>	0
	10 <sup>10</sup>	0
	10 <sup>11</sup>	0
RC-14	10 <sup>6</sup>	0
	10 <sup>7</sup>	0
	10 <sup>8</sup>	0
	10 <sup>9</sup>	0
	10 <sup>10</sup>	0
	10 <sup>11</sup>	0

<sup>a</sup> The number of colony forming units [CFUs] used to inoculate surgical implants.

<sup>b</sup> The percentage of acute surgical infection (Day 3).

**Table 1. The effect of *L. fermentum* RC-14 and *L. rhamnosus* GR-1 on surgical implants in Sprague Dawley rats.** Silicone implants (1cm<sup>2</sup>) were surgically placed in a small dorsal subcutaneous pocket in male rats (Sprague Dawley, 300gm) and inoculated with the indicated number of colony forming units [CFUs] of *L. fermentum* RC-14 or *L. rhamnosus* GR-1. Animals were sacrificed 3 days after surgery. Wound tissue and surgical implants were collected for further analysis.

<b>Table 2</b> The effects of Probiotic strains of <i>Lactobacilli</i> on <i>S. aureus</i> Induced Surgical Implant Infection in Rats		
Bacterial strains	<i>Lactobacilli</i> [CFUs] <sup>a</sup>	Infection (%) <sup>b</sup>
<i>S. aureus</i> [ID50] <sup>c</sup>	-	55
	10 <sup>8</sup>	55
	10 <sup>7</sup>	55
GR-1+ <i>S. aureus</i> [ID50]	10 <sup>8</sup>	44
	10 <sup>9</sup>	44
	10 <sup>10</sup>	44
	10 <sup>11</sup>	55
	10 <sup>8</sup>	44
RC-14+ <i>S. aureus</i> [ID50]	10 <sup>7</sup>	22
	10 <sup>8</sup>	11
	10 <sup>9</sup>	11
	10 <sup>10</sup>	0
	10 <sup>11</sup>	0
<sup>a</sup> Number of <i>Lactobacilli</i> colony forming units (CFUs) used to inoculate the surgical subcutaneous pockets containing the silicone implant <sup>b</sup> Acute surgical infection (Day 3) <sup>c</sup> Dose or number of <i>S. aureus</i> CFUs that caused acute surgical infection in 50% of the rats		

**Table 2. The Effect of Probiotic Strains of *Lactobacilli* on *S. aureus* induced Surgical Implant Infection in Sprague Dawley rats.** Silicone implants (1cm<sup>2</sup>) were surgically placed in a small dorsal subcutaneous pocket in male rats (Sprague Dawley, 300gm) and co-inoculated with the indicated number of colony forming units [CFUs] of *S. aureus* and either *L. fermentum* RC-14 or *L. rhamnosus* GR-1. Animals were sacrificed 3 days after surgery. Acute surgical infection (large fluid-filled capsule containing the surgical implant) was scored and wound tissue and implants collected for further analysis.

**Table 3** The Effects of *L. fermentum* RC-14 on *S. aureus* (SA) induced Acute Surgical Implant Infection in Rats

Bacterial Strains	Day	Infection (%)
SA [ID50]		50
SA [ID50]+RC-14 $10^{11}$ CFUs	2	0
SA [ID50]		50
SA [ID50]+RC-14 $10^{11}$ CFUs	3	0
SA [ID50]		50
SA [ID50]+RC-14 $10^{11}$ CFUs	4	0
SA [ID50]		50
SA [ID50]+RC-14 $10^{11}$ CFUs	5	0

**Table 3. The effect of *L. fermentum* RC-14 on *S. aureus* (SA) induced surgical implant infection: an extended Acute Time Course study.** Silicone implants ( $1\text{cm}^2$ ) were surgically placed in a small dorsal subcutaneous pocket in male rats (Sprague Dawley, 300gm) and inoculated with the indicated number of colony forming units [CFUs] of *L. fermentum* RC-14 and/or *S. aureus*. [ID50]. Animals were sacrificed and signs of acute surgical infection assessed on the indicated days after surgery.

The lactobacillus compositions of the present invention at various doses ( $10^3$ - $10^{11}$ ) significantly reduced surgical implant infection caused by of *S. aureus* (See Figures 1A-1B and 6).

EXAMPLE 2Identification of Lactobacillus by-products-collagen binding protein

5 Proteins usually do not act alone and are often complexed with other important proteins. For example, the transcription and replication machinery, ribosomes and the cytoskeleton are all multiprotein complexes that control fundamental cellular processes.

10 Identifying and characterizing the proteins present within these complexes is paramount to understanding how they function normally. Recent advances in biological mass spectroscopy were used to analyze trace concentrations of proteins in their native environments.

15 The SELDI (Surface Enhanced Laser Desorption/Ionization) ProteinChip technology of Ciphergen Biosystems combines the analytical sensitivity of mass spectroscopy ( $10^{-15}$  to  $10^{-12}$  range) with novel surface chemistry capable of either general or selective capture of proteins from

20 small crude biological samples.

Using this technology, lactobacilli components were identified that promoted bacterial homeostasis in a wound. This identification was possible because lactobacilli and other probiotic organisms out-competed

25 the pathogens, such as *Staphylococcus aureus* and *S. epidermidis*, and because the pathogens triggered Lactobacillus to produce a bioactive compound, i.e. a collagen binding protein that either directly or indirectly (immune stimulation) inhibited the growth of

30 the pathogens. The effect was also aided by the probiotic organisms acting as chemotactic agents for immune defenses, such as neutrophils, macrophages, lymphocytes, antibodies and complement.

EXAMPLE 3

SELDI (surface enhanced laser desorption/ionization) was used to separate, detect and analyze native proteins at the femtomole level without using labeling or time consuming biochemical analytical systems. The SELDI system was used to quickly and accurately determine whether clinically important strains of lactobacilli expressed collagen binding proteins.

Four *Lactobacillus* strains were tested. *L. fermentum* RC-14 was selected because of its potent biosurfactant inhibitory activity against many urogenital pathogens. *L. rhamnosus* GR-1 and 36 also produced biosurfactant, and were also inhibitory to enterococci.

The organisms were grown in MRS broth overnight, harvested and the biosurfactant isolated by incubating the organisms for two hours at room temperature.

SELDI System. The resultant data showed the presence of several collagen binding proteins in the RC-14 biosurfactant preparation tested with calf skin and human placental collagen, particularly at 1.9, 4.7, 9.4, 14.2, 26 and 37 kDa (Figures 2 and 3). Strains GR-1, RC-14 and 36 contained both a 26 kD and 36 kD protein. Further analysis of the biosurfactants showed the presence of sixteen amino acids present in varying amounts. (Table 4)

**TABLE 4**  
**AMINO ACID COMPOSITION OF HYDROLYZED**  
**LACTOBACILLUS BIOSURFACTANTS**

Bio-surfactant	**AMINO ACID COMPOSITION (MOLE %)															
	Asx*	Thr	Ser	Glx*	Gly	Ala	Val	Met	Ile	Leu	Phe	Tyr	His	Lys	Arg	Pro
36***	8.23	3.6	2.98	10.59	8.4	33.98***	5.41	1.07	3.58	5.82	1.29	1.97	1.27	5.9	2.39	3.52
GR-1	10.3	7.0	12.3	18.4	18.7	7.86	-	-	2.02	10.7	-	-	4.1	-	1.05	6.94
RC-14	10.4	4.67	5.81	12.5	10.1	8.91	6.19	1.02	3.24	9.5	2.67	3.54	3.64	7.6	5.76	4.52

\* Sample preparation resulted in the deamination of asparagine and glutamine into aspartic acid and glutamic acid, respectively.

\*\* Due to analysis conditions cysteine and tryptophan could not be accurately quantified.

\*\*\* The unlikely high values indicated the presence of free alanine in the sample.

**EXAMPLE 4**

Lactobacilli were rapidly detected from surgical implant associated specimens via intergenic 16S-23S Ribosomal spacer PCR analysis using specific primers of *L. fermentum* RC14. The following method was employed:

Lactobacilli isolates were cultured at 37°C for 48 hours on an LBS plate in anaerobic chamber. One loop of bacteria colonies was picked from the LBS plate and suspended in 1 ml of d<sub>2</sub>H<sub>2</sub>O, then centrifuged for 1 min at 12,000 rpm. 200 µl of InstaGene matrix (Bio-Rad) was added to the pellet and incubated at 56°C in a water bath for 30 min. The pellet was vortexed at high speed for 10 seconds keeping the sample in the boiling waterbath for 8 min. The sample was vortexed at high speed again and spun at 12,000 rpm for 3 min. The chromosomal DNA was stored at -20°C until used.

Optimal PCR conditions for different strains of *Lactobacillus* were established by using two universal primers from *E. coli*. The DNA fragment containing the spacer regions between 16S rRNA and 23S rRNA genes of RC-14 strains was amplified by using PCR with two universal primers A1 and B1 from *E. coli*. The 5' primer, 5'AGTCGTAACAAGGTAAGCCG3' (SEQ ID NO:7) corresponds to a conserved sequence motif from the 3' end of 16S rRNAs [Primer A1, position 1493 - 1513 (*Escherichia coli* 16S rRNA numbering)] and the 3' primer, 5'C T/C A/G T/C TGCCAAGCATCCACT3' (SEQ ID NO:8) was deduced from an alignment of the 13 23S 5' sequences [primer B1, position 23 - 43 (*Escherichia coli* 23S rRNA numbering)], respectively. DNA templates (1.6 ug, 40 µl) were amplified in a 100 µl reaction volume that contained 2.5 u *Taq* polymerase (Boehringer Mannheim), 100 ng of each of



the primers, 4 mM MgCl<sub>2</sub>, 0.2 mM of each of the four dNTPs (Pharmacia Biotech), 10 mM Tris-Cl (PH 8.0), 50 mM KCl and 1% (v/v) Triton X-100. Reaction mixtures were overlaid with 100 µl mini oil (liquired paraffin, VWR) and preheated at 95° for 5 min. Amplification was carried out in a AMPLITRON II Thermolyne for 40 cycles. Each amplification cycle was as follows: 30 seconds at 95°C (denaturation), 1 min. at 40°C, 45°C or 50°C. The optimal annealing temperature was 40°C for RC-14, and 1 min at 72°C (extension). Post dwell 7 min. at 72°C. Controls were included in each set of amplifications. The controls consisted of a reaction mixture with no DNA template added.

Analysis of the degree and the specificity of PCR products was conducted by 2.5% agarose gel in 1x TAE buffer, running at 70 Volts for 2½ hours. The gel was stained with ethidium bromide and photographed under UV light. DNA fragment sizes were compared with the 100bp DNA Molecular Weight (Gibco-Life Tech.) There were two PCR bands for RC14 (Band 1: 220bp and Band 2: 180bp).

A QIAquick Gel Extraction Kit (Qiagen, Mississauga, Ontario) for extraction of DNA fragments 70bp-10kb from standard agrose gel in TAE or TBE buffer was used to purify PCR bands.

Each of the two PCR DNA fragment bands were excised from the agarose gel with a scalpel and the gel slice was weighed. The protocol of QIAquick Gel Extraction Kit was then followed. The Kit system combined the spin-column with the silica-gel membrane. The DNA band was dissolved completely with solubilization buffer in 50°C for 10 min. DNA adsorbed to the silica membrane in the high salt conditions. Pure DNA was eluted with

Tris buffer (PH 8.0). This pure PCR product was stored at -20°C for later use.

Each PCR band product was ligated into pGEM-T vector (Promega). Each pGEM-T vector was transformed into E. coli JM 109 high efficiency competent cells by using Transformation Aid (MBI Fermentas Inc.) on the LB plate with 50 ug/ml ampicillin. Several white colonies or light blue colonies were selected as positive colonies which contained the PCR insert. Colonies were cultured on the LB-ampicillin plate. Each plate contained 32 different colonies. Colonies were cultured with LB-ampicillin broth. One part of culture was frozen quickly by using liquid nitrogen and was kept at -80°C. Another part of culture was used for further miniprep of plasmid DNA. The remainder of culture was kept at 4°C.

The QIAprep Spin Miniprep Kit (Qiagen, Mississauga, Ontario) was used to prepare plasmid DNA. Each of two PCR products was automatically sequenced by using T7 & SP6 promoter primers with two directions. Analysis of sequence was performed using the sequence analysis software package - DNA Star program.

DNA templates (1.6 ug, 40 µl) were amplified in a 100 µl reaction volume that contained 2.5 u Taq polymerase (Boehringer Mannheim), 100 ng of each of the primer, 4 mM MgCl<sub>2</sub>, 0.2 mM of each of the four dNTPs (Pharmacia Biotech), 10 mM Tris-Cl (PH 8.0), 50 mM KCl, and 1% (v/v) Triton X-100. Reaction mixtures were overlaid with 100 µl mini oil and preheated at 95°C for 5 min. Amplification was carried out in a AMPLITRON II Thermolyne for 25 cycles. Each amplification cycle was as follows: 30 seconds at 95°C (denaturation), 1 min. at 60°C (annealing), and 1 min. at 72°C (extension). Post dwell 7

min. at 72°C. Controls were included in each set of amplifications.

Verification and confirmation of detection of *Lactobacillus fermentum* RC-14 and *S. aureus* was performed using a traditional API 50 commercial biochemistry test (API Systems, La Balme, Les Grottes, France) and PCR primer. (See Figures 5B & 5C)

**WHAT IS CLAIMED IS:**

1. A method for inhibiting wound infections in a mammal in need thereof which comprises administering to said mammal a therapeutically effective amount of probiotic organisms to an infected wound site.

2. The method of Claim 1 wherein said probiotic organism is a *Lactobacillus*.

3. The method of Claim 2 wherein said *Lactobacillus* is selected from the group consisting of *L. rhamnosus*, *L. acidophilus*, *L. fermentum*, *L. casei*, *L. reuteri*, *L. crispatus*, *L. plantarum*, *L. paracasei*, *L. jensenii*, *L. gasseri*, *L. cellobiosis*, *L. brevis*, *L. delbrueckii*, *L. helveticus*, *L. salivarius*, *L. collinoides*, *L. buchneri*, *L. rogosae*, or *L. bifidum*.

4. The method of Claim 1 further comprising the administration of a therapeutically effective amount of a prebiotic.

5. The method of Claim 1 further comprising the administration of a therapeutically effective amount of a collagen binding protein.

6. A method for inhibiting biofilm formation in a mammal in need thereof which comprises coating at least part of a surgically implantable device with a therapeutically effective amount of a probiotic organism.

7. The method of Claim 6 wherein said probiotic

organism is a *Lactobacillus*.

8. The method of Claim 7 wherein said  
Lactobacillus is selected from the group consisting of *L.*  
5 *ramnosus*, *L. acidophilus*, *L. fermentum*, *L. casei*, *L.*  
*reuteri*, *L. crispatus*, *L. plantarum*, *L. paracasei*, *L.*  
*jensenii*, *L. gasseri*, *L. cellobiosus*, *L. brevis*, *L.*  
*delbrueckii*, *L. helveticus*, *L. salivarius*, *L. collinoides*,  
10 *L. buchneri*, *L. rogosae*, and *L. bifidum*.

9. A pharmaceutical composition suitable for  
preventing wound infections in mammals which comprises a  
therapeutically effective amount of a probiotic organism  
and a pharmaceutically acceptable carrier.

10. A method for stimulating an immune response  
against infection in a mammal in need thereof which  
comprises administering to said mammal a therapeutically  
effective amount of probiotic organisms.

11. The method of Claim 10 wherein said  
probiotic organism is a *Lactobacillus*.

12. The method of Claim 11 wherein said  
Lactobacillus is selected from the group consisting of *L.*  
25 *ramnosus*, *L. acidophilus*, *L. fermentum*, *L. casei*, *L.*  
*reuteri*, *L. crispatus*, *L. plantarum*, *L. paracasei*, *L.*  
*jensenii*, *L. gasseri*, *L. cellobiosus*, *L. brevis*, *L.*  
*delbrueckii*, *L. helveticus*, *L. salivarius*, *L. collinoides*,  
30 *L. buchneri*, *L. rogosae*, or *L. bifidum*.

13. The method of Claim 10 further comprising

the administration of a therapeutically effective amount of a prebiotic.

5           14. A method for reducing the risk of medical device associated infections in a mammal which comprises coating a medical device with a therapeutically effective amount of probiotic organism and providing said device to said mammal.

10           15. The method of Claim 14 wherein said probiotic organism is a *Lactobacillus*.

15           16. The method of Claim 15 wherein said *Lactobacillus* is selected from the group consisting of *L. rhamnosus*, *L. acidophilus*, *L. fermentum*, *L. casei*, *L. reuteri*, *L. crispatus*, *L. plantarum*, *L. paracasei*, *L. jensenii*, *L. gasseri*, *L. cellobiosis*, *L. brevis*, *L. delbrueckii*, *L. helveticus*, *L. salivarius*, *L. collinoides*, *L. buchneri*, *L. rogosae*, or *L. bifidum*.

20           17. The method of Claim 14 further comprising the administration of a therapeutically effective amount of a prebiotic.

25           18. A method for the inhibition of pathogenic colonization in a host comprising the administration of a probiotic organism cultured to induce the production of a collagen binding protein.

30           19. A method of reducing the occurrence of pathogenic bacteria in a host to less than about 100,000 bacteria per cubic cm per gram of tissue comprising

administration of about  $10^3$  to about  $10^{11}$  of a probiotic organism to a wound infection site.

5

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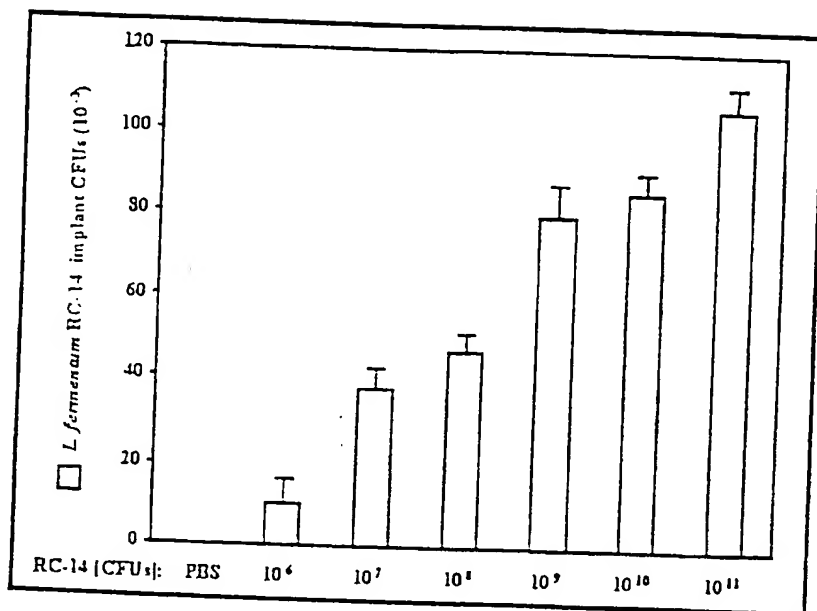


FIGURE 1A



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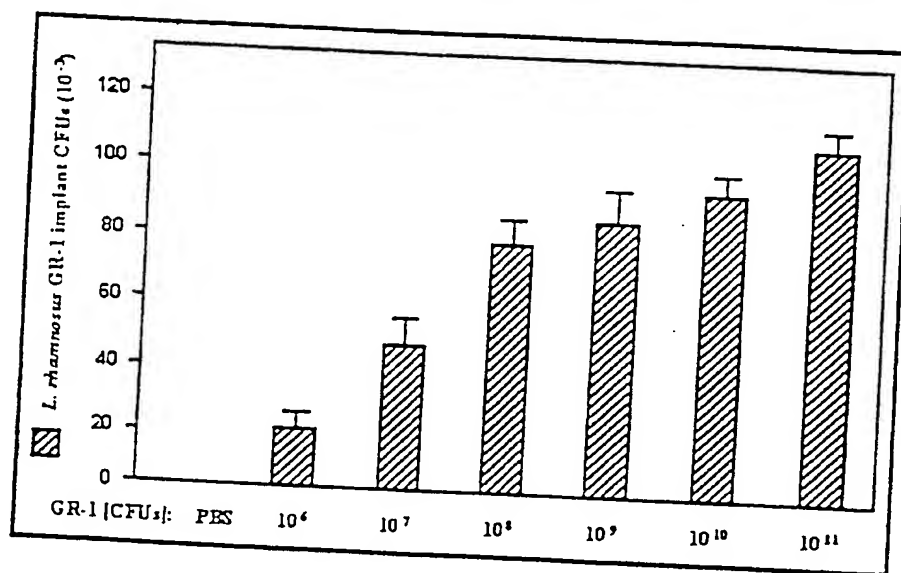


FIGURE 1B

3/10

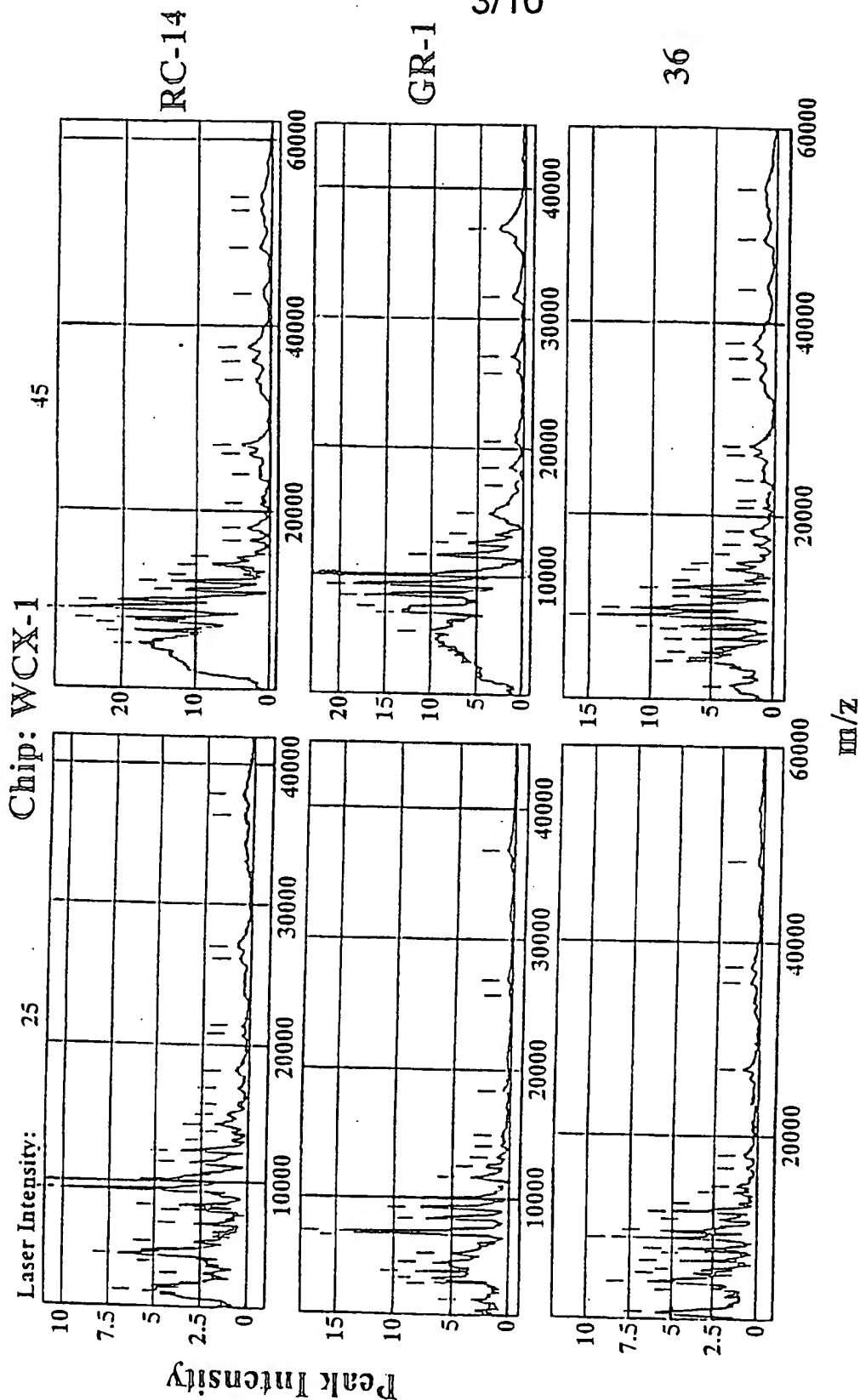


FIGURE 2

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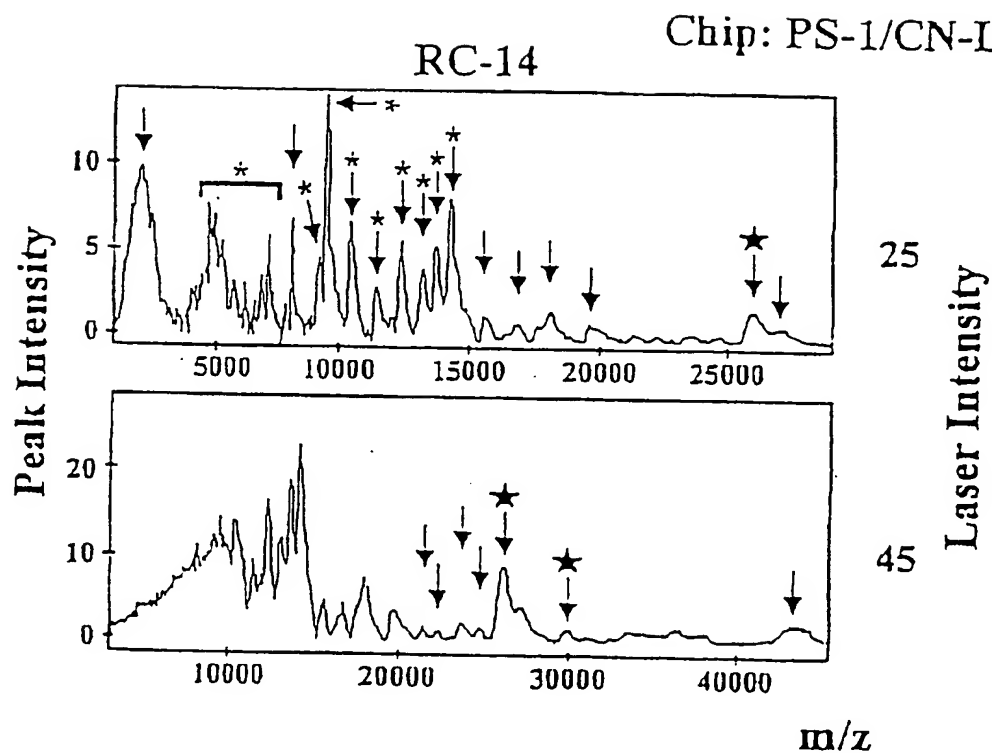


FIGURE 3

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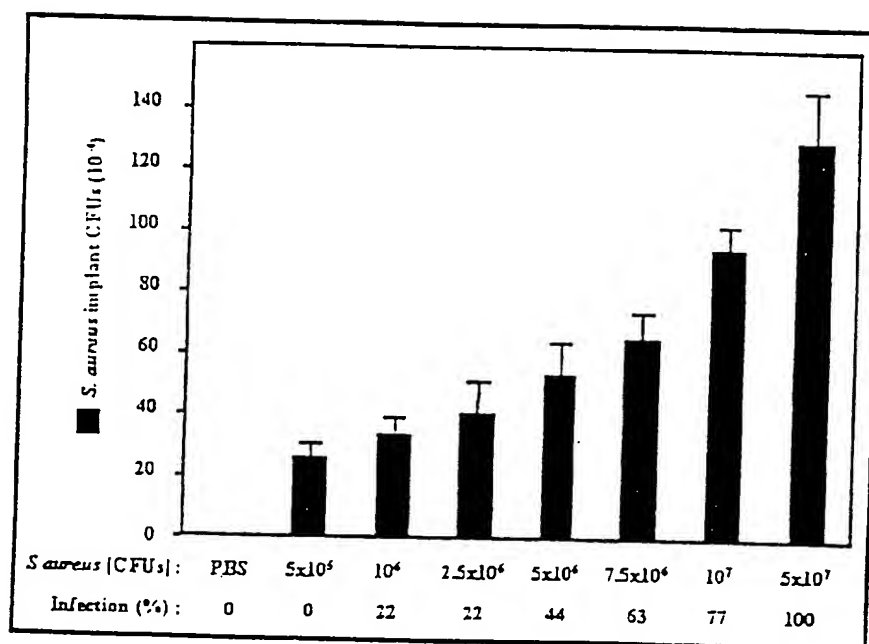


FIGURE 4

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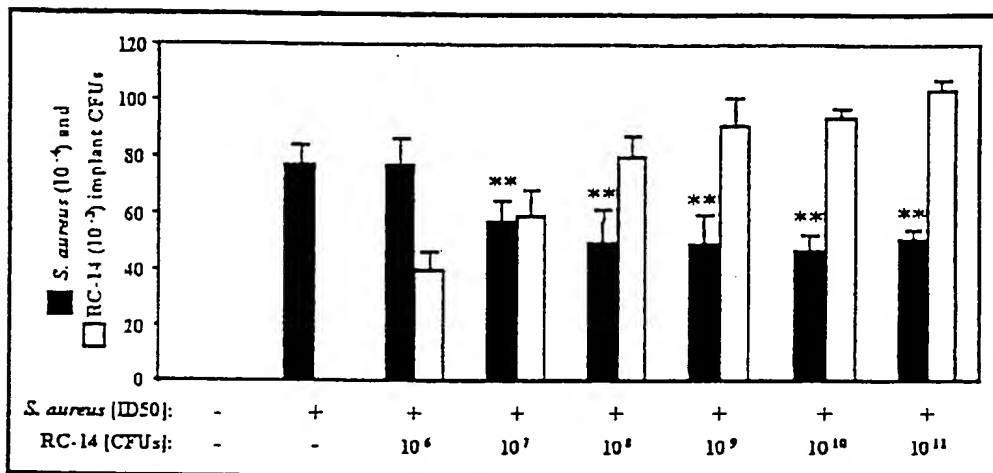


FIGURE 5A

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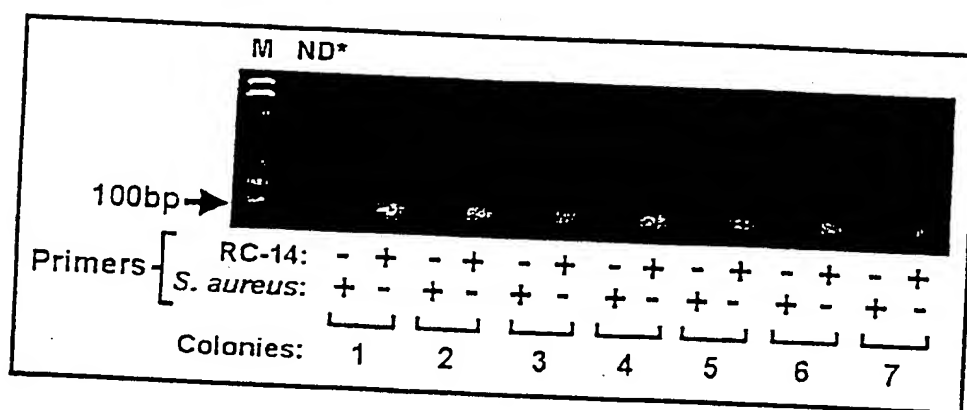


FIGURE 5B

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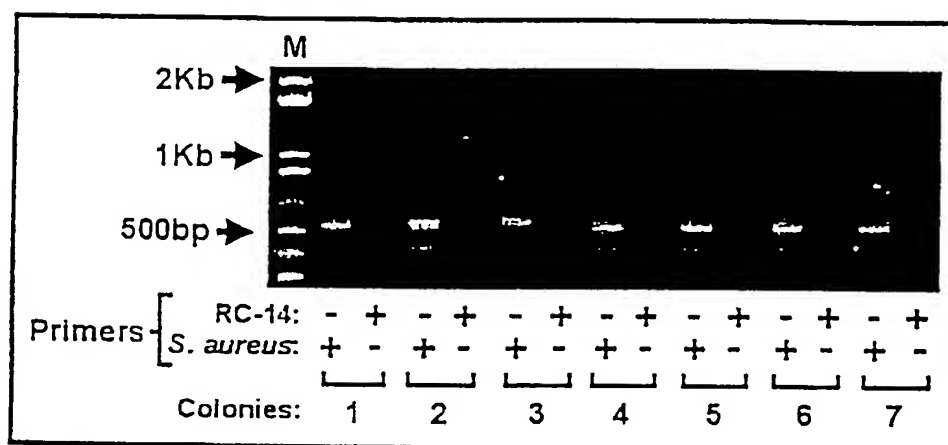


FIGURE 5C

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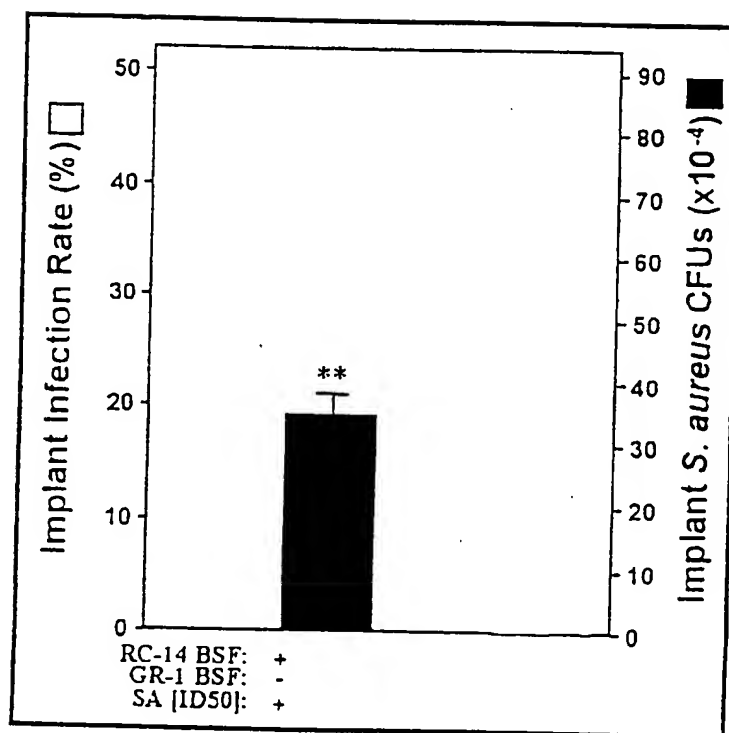


FIGURE 6



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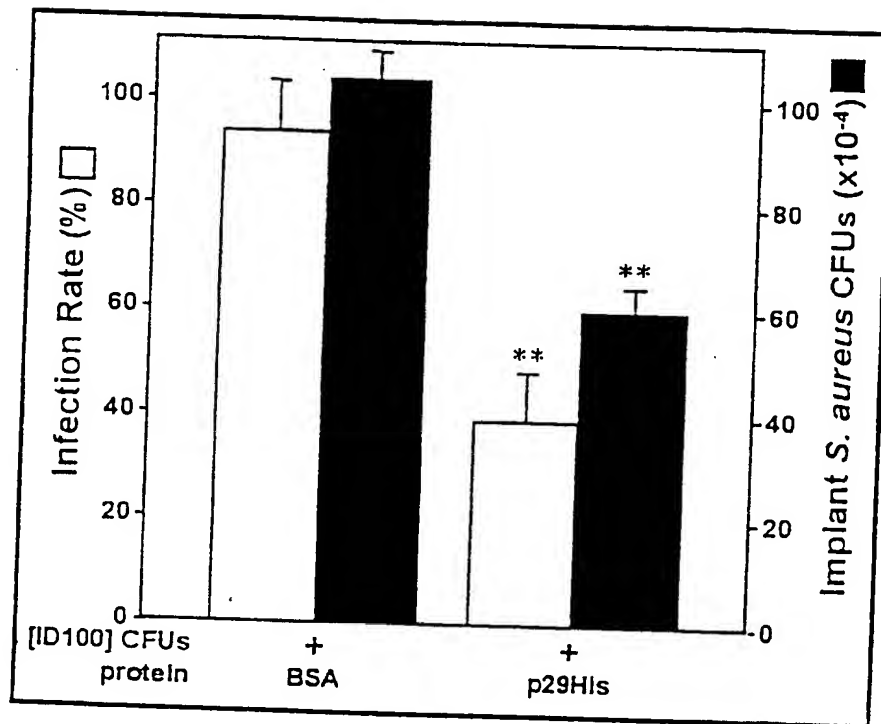


FIGURE 7

(19) World Intellectual Property Organization  
International Bureau



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(10) International Publication Number  
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- (25) Filing Language: English
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- (30) Priority Data:  
60/135,954 25 May 1999 (25.05.1999) US
- (71) Applicants and  
(72) Inventors: REID, Gregor [CA/CA]; 71 Chesham Avenue, London, Ontario N6G 3V1 (CA). BRUCE, Andrew, W. [CA/CA]; Apt. 507, 235 St. Claire Avenue, West, Toronto, Ontario M4V 1R4 (CA).
- (74) Agents: ZAHL, Adrian et al.; McFadden, Fincham, 225 Metcalfe Street, Suite 606, Ottawa, Ontario K2P 1P9 (CA).
- (81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:  
— With international search report.
- (88) Date of publication of the international search report:  
8 March 2001
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 00/71139 A3

(54) Title: LACTOBACILLUS COMPOSITIONS AND METHODS FOR PREVENTING WOUND INFECTIONS AND BIOFILM FORMATION ON IMPLANTABLE SURGICAL DEVICES

(57) Abstract: This invention relates to probiotic compositions and their by-products and methods of employing said compositions for inhibiting, treating or preventing wound infections and biofilm formation on implantable surgical devices.

# INTERNATIONAL SEARCH REPORT

Inter. Application No

PCT/CA 00/00611

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 A61K35/74 A61P31/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, CHEM ABS Data, EMBASE, SCISEARCH

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RASIC J LJ & KURMANN J A: "Yoghurt" SCIENTIFIC GROUNDS, TECHNOLOGY, MANUFACTURE AND PREPARATIONS, COPENHAGEN, TECHNICAL DAIRY PUBLISHING, DK, 1978, pages 120-137, XP002142806 the whole document, especially pages 131-136	1-4, 9, 19
X	DE 27 38 652 A (SEIKEN KAI FOUNDATIONAL JURIDI) 15 March 1979 (1979-03-15) the whole document  -/--	1-4, 9, 19

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

\*A\* document defining the general state of the art which is not considered to be of particular relevance

\*E\* earlier document but published on or after the international filing date

\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*Z\* document member of the same patent family

Date of the actual completion of the international search

8 December 2000

Date of mailing of the international search report

15/12/2000

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Stein, A

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 00/00611

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>HORIKAWA Y: "EFFECTS OF LACTOBACILLUS-CASEI CONTAINING OINTMENT ON THE HEALING AND PROTECTION AGAINST OPPORTUNISTIC INFECTION OF THERMAL INJURY WOUNDS IN MICE" HIROSHIMA JOURNAL OF MEDICAL SCIENCES, vol. 35, no. 1, 1986, pages 1-14, XP000961244 ISSN: 0018-2052 the whole document, especially page 1 column 2 lines 4-11, page 2 column 1 line 29-column 2 line 7, page 12 column 2 line 17-page 13 column 2 line 1 and figures 8-10</p>	1-4, 9-13,19
X	<p>WO 97 14802 A (TNO ;LEER ROBERT JAN (NL); POUWELS PIETER HENDRIK (NL); CONWAY PAT) 24 April 1997 (1997-04-24) page 1, line 22 - line 31 page 4, line 27 - line 30 page 10, line 31 -page 11, line 3 page 18, line 11 - line 33 page 25, line 13 - line 16 claims 1-39</p>	1-3,5,18
X	<p>HAWTHORN L A ET AL: "EXCLUSION OF UROPATHOGEN ADHESION TO POLYMER SURFACES BY LACTOBACILLUS-ACIDOPHILUS" JOURNAL OF BIOMEDICAL MATERIALS RESEARCH, vol. 24, no. 1, 1990, pages 39-46, XP000961246 ISSN: 0021-9304 the whole document, especially page 44 lines 27-33</p>	6-8, 14-16
X	<p>US 5 804 179 A (REID GREGOR ET AL) 8 September 1998 (1998-09-08) the whole document</p>	6-8, 14-17
X	<p>WO 98 23727 A (BIO K &amp; INTERNATIONAL INC ;LUQUET FRANCOIS MARIE (FR)) 4 June 1998 (1998-06-04) page 4, line 15 - line 20 page 11, line 1 - line 14 claims 1-6,8-11,15-22</p>	10-13
X	<p>US 5 645 830 A (REID GREGOR ET AL) 8 July 1997 (1997-07-08) the whole document</p>	14-17
	-/--	

# INTERNATIONAL SEARCH REPORT

Inter. nal Application No

PCT/CA 00/00611

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GRISTINA A G: "BIOMATERIAL-CENTERED INFECTION MICROBIAL ADHESION VERSUS TISSUE INTEGRATION" SCIENCE (WASHINGTON D C), vol. 237, no. 4822, 1987, pages 1588-1595, XP000971033 ISSN: 0036-8075 cited in the application the whole document	6,14
T	HEINEMANN CHRISTINE ET AL: "Purification and characterization of a surface-binding protein from Lactobacillus fermentum RC-14 that inhibits adhesion of Enterococcus faecalis 1131." FEMS MICROBIOLOGY LETTERS, vol. 190, no. 1, 2000, pages 177-180, XP000971010 ISSN: 0378-1097 the whole document, especially page 179 column 1 line 34-column 2 line 5	5,18

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## Continuation of Box I.2

Present claims 1,4-6,9,10,13,14,17-19 relate to organisms defined by reference to a desirable property, namely their probiotic activity, claims 4, 13 and 17 further relate to a compound defined by its prebiotic activity and claims 5 and 18 further relate to a protein defined by its collagen binding property. However the claims lack any structural and essential characteristics of the organisms, compounds and proteins. The claims cover all organisms having this probiotic activity, all the compounds having prebiotic activity and all the proteins which are able to bind to collagen, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for only a very limited number of such organisms, compounds and proteins. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the organisms, compounds and proteins by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the organisms as defined on page 7 line 27-page 8 line 2, page 13 line 29-page 14 line 2, page 15 line 11-page 16 line 11 and claims 2,3,7,8,11,12,15 of the application, those parts relating to the compound as defined on page 18 lines 4-11 of the application and those parts relating to the proteins as defined on page 17 lines 5-18 of the application. In addition the general concept of the claims has been searched (general terms of probiotic, prebiotic and collagen-binding protein).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 00/00611

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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